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TI Nonradioactive labeling of synthetic ***oligonucleotide*** probes with terminal deoxynucleotidyl transferase.
CM Erratum in: Anal Biochem 1988 Sep;173(2):469
AU Kumar A; Tchen P; Roullet F; Cohen J
CS Station de Recherches de Virologie et d'Immunologie INRA, Thiverval-Grignon, France.
SO ANALYTICAL BIOCHEMISTRY, (1988 Mar) 169 (2) 376-82.

TI Use of bioluminescence in nucleic acid hybridization reactions.
AU Balaguer P; Terouanne B; Boussioux A M; Nicolas J C
CS I.N.S.E.R.M. Unite 58, Montpellier, France.
SO JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE, (1989 Jul) 4 (1) 302-9.

TI The preparation of polyamide- ***oligonucleotide*** probes containing multiple non-radioactive labels.
AU Haralambidis J; Angus K; Pownall S; Duncan L; Chai M; Tregear G W
CS Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria, Australia.
SO NUCLEIC ACIDS RESEARCH, (1990 Feb 11) 18 (3) 501-5.

Thank you-

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Use of Bioluminescence in Nucleic Acid Hybridization Reactions

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Luminescence reactions can be used to detect specific nucleic acid sequences hybridized with a nucleic probe. Different labels such as cytidine sulphone, fluorescein, and biotin can be incorporated into DNA or oligonucleotide molecules and detected by antibody or avidin conjugates coupled to glucose-6P dehydrogenase.

On supports such as nitrocellulose filters, sensitivity is not greatly increased using luminescence, but detection is rapid and easy to perform using polaroid film. Moreover, hybridization can be performed with different labelled probes on the same sample.

In solution, luminescence can be used to monitor sandwich reactions. The method is less sensitive than detection on filters but can easily be automated. The performance of these assays can be increased considerably by enzymatic amplification of the target catalysed by a thermostable polymerase.

Keywords: Bioluminescence; dehydrogenase; nucleic acid; hybridization; amplification

INTRODUCTION

Many methods of DNA labelling have been described (Leary *et al.*, 1983; Tchen *et al.*, 1984; Forster *et al.*, 1985; Oser *et al.*, 1988; Lebacq *et al.*, 1988). Phosphatase and peroxidase are the enzymes most commonly used (Jablonsky *et al.*, 1986; Renz and Kurtz, 1984) in this kind of experiment. These enzymes can be detected by the formation of a chromogen or by luminescent reactions (Matthews *et al.*, 1985; Hauber and Geiger 1988). We have found that luminescent assay with phosphatase is very problematic since the substrate, luciferin phosphate, is easily hydrolysed in solution. Consequently, we now use glucose-6-phosphate dehydrogenase (G6PDH) as the marker. G6PDH can be detected at low levels using bioluminescence reations catalysed by marine bacterial enzymes. This dehydrogenase has been successfully used as a label in enzyme immunoassays (Térouanne *et al.*, 1986; Carrié *et al.*, 1986) and in the present study we investigated its use for monitoring nucleic acid hybridization reactions. G6PDH was coupled to avidin, streptavidin, or to antibodies, and these conjugates were used to detect biotin or haptens linked to DNA probes. The bioluminescent reaction can be used to detect the dehydrogenase either after nucleic hybridization on nitrocellulose filters or in solution.

MATERIALS AND METHODS

Reagents

Decanal, *S*-acetyl mercaptosuccinic anhydride (SAMSA), and fluorescein isothiocyanate were

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obtained from Sigma. Sephadex G50 medium was purchased from Pharmacia. Glucose-6-phosphate dehydrogenase (G6PDH) of *Leuconostoc mesenteroides* (EC 1.1.1.49) at 559 IU/mg. Glucose-6-phosphate (G6P), nicotinamide-adenine-dinucleotide (NAD), and flavine mononucleotide (FMN) were obtained from Boehringer. Luciferase and FMN oxidoreductase of *Beneckea harveyi* were purified as previously described (Hastings *et al.*, 1978; Jablonski and DeLuca, 1977).

Preparation of enzyme conjugates

Coupling procedure. For modification, protein (1 mg) was dissolved in 300 µl of 0.1 mol/l sodium phosphate buffer, pH 7.5, and incubated with 6 µl of SAMSA (10^{-1} mol/l in acetonitrile) for one hour. Thioester links were hydrolysed by incubation with 30 µl of hydroxylamine (1 mol/l, pH 7.5) and 5 µl of DTE (0.1 mol/l) for 20 min. The thiolated protein was isolated by gel filtration on Sephadex G 25 (PD 10) in 20 mmol/l sodium phosphate, pH 6.4, and 5×10^{-3} mol/l EDTA.

G6PDH (1.5 mg) in 500 µl of 0.1 mol/l sodium phosphate (pH 7.5) was incubated with 25 µl of SMCC (10^{-2} mol/l in DMF) in the presence of 10^{-4} mol/l NADP and 3×10^{-3} mol/l G6P to reduce enzyme inactivation. The reaction mixture was incubated for 45 min at room temperature and passed through a Sephadex G 25 column equilibrated with 20 mmol/l sodium phosphate (pH 6.4) and 5×10^{-3} mol/l EDTA.

Thiolated protein and maleimide G6PDH fractions were collected, concentrated on Amicon 30 and incubated for 18 h at 4 °C. The reaction mixture was then incubated with 4 mmol/l β-mercaptoethanol for 30 min and subjected to chromatography on Superose 12 (FPLC) in 0.1 mol/l sodium phosphate (pH 7.4), 2×10^{-3} mol/l β-mercaptoethanol, and 2×10^{-3} mol/l EDTA. The conjugate fractions were stored at 20 °C in 1% BSA and 20% glycerol.

Bioluminescent assay of enzyme conjugates. G6PDH conjugate (50 µl, 0.1 mIU) was incubated for 30 min with 100 µl of adsorbent suspension (30 mg/ml). Adsorbent was synthesized by coupling 6-aminohexyl biotin (25 nmol), luciferase (5 mg), and oxidoreductase (1 IU) to 1 g of Sepharose. Luminescence was measured after addition of luminescent reagent (10^{-3} mol/l NAD, 3×10^{-3} mol/l G6P, 3×10^{-3} mol/l sodium pyruvate, 10^{-5} mol/l FMN, 6×10^{-5} mol/l decanal and 30 mIU/ml LDH in phosphate buffer 0.1 mol/l). Non specific binding was obtained by incubating the avidin or streptavidin conjugate (0.1 mIU) with an excess of biotin (0.1 µg).

DNA labelling

Phage λ was labelled with biotin by nick-translation (BRL Kit), substituting biotin–dUTP for dTTP, or using photobiotin (Vector Laboratories). DNA was sulphonated using reagents from Orgenics Ltd (Yavne, Israel).

Biotinylated DNA was recovered by gel filtration on a Sephadex G 50 column equilibrated with 0.1% SDS. Aliquots of the DNA eluted from the column were spotted onto nitrocellulose membranes and the presence of biotin bound to the DNA was confirmed by labelling with streptavidin–alkaline phosphatase from BRL (blue gene) or by our dehydrogenase conjugates. The biotinylated DNA fractions were used without further purification.

Sulphonated DNA did not require purification and these probes were used directly after dilution in hybridization buffer.

Labelling of oligonucleotides

Oligonucleotides were enzymatically labelled by biotin dUTP using deoxynucleotide terminal transferase (Kumar *et al.*, 1988). Two to four biotin dUTP can be incorporated per mole of oligonucleotide. Biotin or haptens such as fluorescein can also be linked to the oligonucleotide by chemical reaction. The oligonucleotide was synthesized with an amino group at the 5′ end and coupled to biotin *N*-hydroxysuccinimide ester or fluorescein isothiocyanate as previously described by Agrawal *et al.* (1986).

Dot-blot hybridizations

Denatured nucleic acid in 1.5 mol/l NaCl, 0.15 mol/l sodium citrate buffer, pH 6.9 (10 × SSC) with 0.2 mg/ml denatured salmon sperm DNA was spotted onto nitrocellulose filters. Filters were allowed to dry at room temperature and baked for 1 h at 100 °C. They were then stored desiccated at room temperature until use.

The filters were prehybridized in 2X hybridization buffer* at a temperature of 64 °C in a water bath. The DNA probe was heated in a boiling water bath for 10 min and added to the 1X hybridization buffer solution at 1 µg/ml and thoroughly mixed.

The filters were hybridized overnight at 64 °C, then washed for 10 min with 0.2X SSC, 0.2% Tween, twice at room temperature and twice at 60 °C.

Hybridization in solution

Denatured DNA was allowed to hybridize with the denatured DNA probes (10 ng) in 40 µl of a solution containing 0.6 mol/l NaCl, 100 mmol/l phosphate, pH 7.5, 1 mmol/l EDTA and 0.1% SDS at 65 °C in Eppendorf tubes under a layer of mineral oil to avoid evaporation.

DNA amplification

DNA amplification was done as described by Kogan *et al.* (1987) in 100 µl of a reaction mixture containing 1 µg of genomic DNA, 0.2 mmol/l of dATP, dCTP, dGTP, dTTP, and 1 µmol/l of each labelled oligonucleotide primer in reaction buffer (16.6 mmol/l $(NH_4)_2SO_4$, 67 mmol/l Tris HCl, pH 8.8 at 25 °C, 6.7 mmol/l $MgCl_2$, 10 mmol/l β-mercaptoethanol, 6.7 mmol/l EDTA and 0.17 g/l gelatin). Samples were heated for 5 min at 94 °C to denature the DNA, and briefly cooled to room temperature. Two units of *Taq* polymerase (Cambio) was added to each sample and heated for 2 min at 55 °C for annealing and for 2 min at 70 °C for primer-directed DNA synthesis of a 200 bp sequence. Rounds of 2 min denaturation, 2 min annealing and 2 min synthesis were continued for 30 cycles. About 100 ng of target DNA were synthesized, resulting in one-million-fold amplification.

Detection of labelled DNA immobilized on filters

Filters were incubated in a blocking buffer (50 mmol/l HCl pH 7.5, 150 mmol/l NaCl, 0.5% gelatin, and 0.2% Tween 80), pre-warmed for 1 h at 60 °C and incubated with avidin or streptavidin G6PDH conjugate (100 mUI/ml) in the same buffer. Filters were washed twice for 10 min in 50 mmol/l Tris HCl, pH 7.5, 150 mmol/l NaCl, 0.2% Tween 80, and twice for 10 min in the same buffer without Tween.

For the sulphone probe, the post-hybridization steps were different: we used a blocking buffer containing 20% horse serum, 10% powdered milk and 2X SSC. Filters were incubated in this buffer for 1 hr at room temperature, and incubated for 1 h with the anti-sulphonated DNA antibody (dilution 1/250) at room temperature with gentle agitation. They were then washed with 0.1% Brij 35, 500 mmol/l NaCl, and incubated for 1 h with the mouse anti-γ-globulin antibody linked to G6PDH, at a concentration of 100 mUI/ml. Filters were again washed with the Brij buffer and then twice with Tris HCl 100 mmol/l, pH 7.5, NaCl 150 mmol/l at room temperature.

Bioluminescent detection.

Quantitative determination. After the last washes, the filters were cut into small squares (0.5 × 0.5 cm) and put into LKB tubes. The bioluminescent reagent was: 10^{-5} mol/l FMN, 6×10^{-5} mol/l decanal, 1 g/l BSA, 3×10^{-3} mol/l NAD, 3×10^{-3} mol/l G6P, 20 µg/ml luciferase, and 1 mIU/ml FMN oxidoreductase in 200 mmol/l phosphate, pH 7. Reagent (200 µl) was injected into each tube and light emission was measured for 10 s with an LKB luminometer or scintillation counter.

Photographic detection. The same reaction was used, but bioluminescent reagent (200 µl) was added to the filter and placed between two pieces of parafilm. The sealed parafilm was inserted into the film holder and left in contact with polaroid film (20,000 ASA) for several minutes.

Using the Hamamatsu video camera, the parafilm bag was placed in the imaging box and light emission was recorded for 10 to 20 min.

Detection of labelled DNA in solution

For detection in solution, DNA must be labelled with two different labels. One label is used to

*Composition of 1X hybridization buffer is 2X SSC, 5X FPGe 25 mmol/l KH_2PO_4, pH 7, 2.5 mmol/l EDTA, 0.2 mg/ml salmon sperm DNA. 10X FPGe contains 0.2% Ficoll, polyvinylpyrolidone, gelatin.

capture the DNA and the second to determine the amount of DNA captured. The labels used were biotin, cytidine sulphone, or fluorescein. DNA was captured using streptavidin immobilized on tubes or magnetic beads (Dynobeads) and a G6PDH-labelled antibody (anti-mouse γ-globulins) was used to determine the amount of bound probes.

Double-labelled DNA was prepared by hybridization with two labelled probes.

The batch procedure used for collection on avidin magnetic beads or streptavidin tubes required incubating the samples with the support for 1 h in 100 µl of 400 mmol/l NaCl, 100 mmol/l phosphate, pH 7. The supernatant was discarded and the beads or tubes washed twice with 1 ml of the buffer. Antibody (anti-sulphonated DNA or anti-fluorescein; 100 µl) was added in 150 mmol/l NaCl, 100 mmol/l phosphate and 1 g/l BSA). After 30 min, reaction tubes were washed twice with buffer and incubated for 30 min with anti-mouse G6PDH conjugate. After two further washes, bioluminescent reagent was added and light emission measured for 10 s.

RESULTS AND DISCUSSION

Properties of G6PDH conjugates

We chose to place thiol groups on avidin and streptavidin using SAMSA since it is known that avidin can be succinylated without drastically affecting its biological properties. Moreover, succinylation has been used to decrease the nonspecific binding often observed with avidin.

Reaction of thiolated avidin or streptavidin with maleimido-G6PDH produced a high yield of conjugates (65%) and after separation by FPLC on Superose 12 we obtained several fractions of increasing molecular weight. We determined the biological activity of these conjugates using a biotin adsorbent. Luminescence was measured when the conjugate was bound to the support and then when an excess of biotin was added to the conjugate (nonspecific binding). Table 1 shows that the main conjugate fraction had the highest molecular weight, but its nonspecific binding, expressed in millivolts per 0.1 mIU of G6PDH, was higher than that of the other fractions. Thus, in our experiment we used conjugates collected before the peak of free G6PDH (fractions 29 to 34, corresponding to conjugate formed with one molecule of enzyme and one molecule of avidin or streptavidin).

Table 1. Activities of G6PDH conjugates eluted from Superose 12. Streptavidin G6PDH conjugate (50 µl; 0.1 mIU) was incubated for 30 min with 100 µl of adsorbent suspension (biotin, luciferase, oxidoreductase, Sepharose) and luminescence, expressed in millivolts, was measured after addition of luminescent reagent

Fractions	G6PDH (IU/ml)	Specific binding	Nonspecific binding	S/NS
23	32.6	33,700	2,790	12.1
27	14.5	39,960	2,600	15.3
30	16.6	28,925	1,310	22.1
33	20.6	18,000	920	19.5
35	45.6	2,900	750	3.8

It is known that streptavidin gives lower nonspecific binding than avidin, but modification of avidin by SAMSA during preparation of the conjugate decreased the nonspecific binding to supports such as polystyrene, or Sepharose. Thus, similar results were obtained with the two conjugates.

The conjugates were used to develop a very sensitive assay of biotin, which does not require a separation step. The principle of the method has previously been described for immunological assays (Térouanne *et al.*, 1986). Less than one femtomole of conjugate was used per tube and as shown in Fig. 1, less than 0.1 femtomole of biotin can be detected with a 30-min incubation.

Use of G6PDH conjugates to detect DNA on nitrocellulose filters

As shown in Table 2, the detection limit obtained with G6PDH streptavidin, measuring light emission with a LKB luminometer, is of the same order as that obtained with phosphatase conjugates. However, quantitative results can be obtained and the measurement can be carried out in several seconds. A good linear relationship is obtained (Fig. 2) and detection is limited essentially by nonspecific binding of the label to the filter. This method is not very handy using the LKB luminometer, since the filter must be cut and placed in separate cuvettes. A multiwell

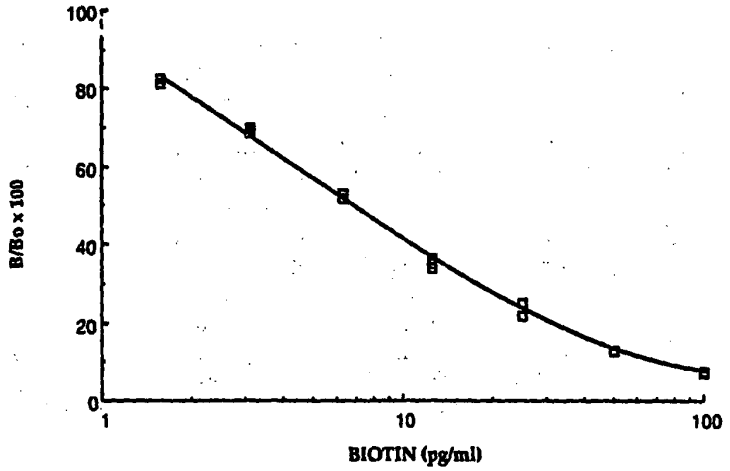

Figure 1. Standard curve of biotin assay obtained using streptavidin-G6PDH conjugate (1 femtomole) and Sepharose coupled to biotin and bioluminescent enzymes

luminometer that can read luminescence from the filter is more adapted to this kind of experiment.

Photographic determination proved to be very useful since the process was rapid and the sensitivity high enough to detect 0.6 pg of target DNA. The method discriminated between high- and low-emitting spots since the difference was enhanced by the high contrast of the film (Fig. 3). Hybridization with a 17-mer oligonucleotide with 0, 1, 2 or 3 base mismatches yielded larger differences with photographic film than with the colorimetric procedure using alkaline phosphatase label.

For rapid and quantitative determination, photon counting camera remains the most useful and sensitive way of measuring luminescence on filters or under a microscope for *in situ* hybridization. Fig. 4 shows results obtained with four samples obtained by polymerase amplification and hybridized with a 17-mer oligonucleotide. The difference in intensity was easily measured and the sensitivity could be adapted to each sample. The detection limit was very low since less than 0.1 attomole of G6PDH was detected after a 20-min exposure.

Table 2. Detection limits obtained with various systems. Results are expressed in programs of phage λ labelled by nick translation with 11 dUTP biotin

Systems	Phosphatase	G6PDH		
	colorimetric	Counting	Films	Video
Probe alone	0.2	0.1	0.2	<0.1
Probe hybridized	0.6	0.4	0.6	0.2

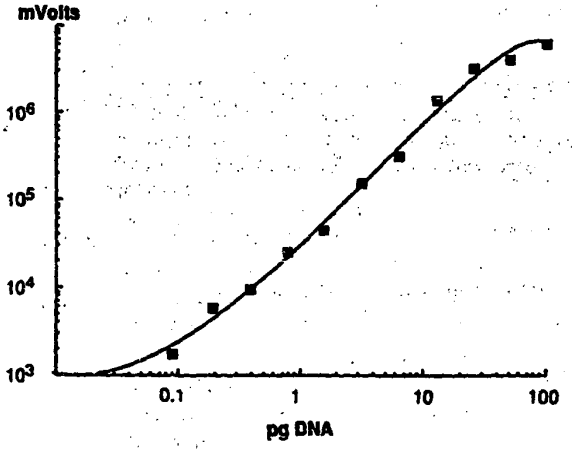

Figure 2. Detection by bioluminescence of biotinylated DNA immobilized on nitrocellulose filter

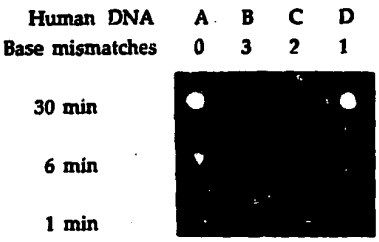

Figure 3. Photographic detection of amplified DNAs hybridized with biotinylated 17-mer oligonucleotide

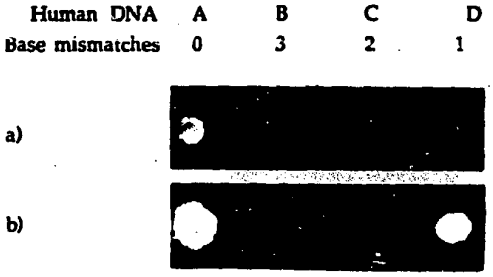

Figure 4. Detection of amplified DNAs by a photon counting camera. (a) Photon density. (b) Light intensity

Detection of hybrids in solution

We studied the performances of the bioluminescent detection of dehydrogenase to determine the amount of double-labelled DNA obtained after hybridization or amplification reactions. Double-labelled DNA was prepared by several methods: double labelling of DNA by sulphonation and biotinylation, by hybridization, or by amplification using labelled oligonucleotides. The molecules were bound to avidin- or streptavidin-coated beads or tubes.

Figure 5 shows results obtained with a double-labelled DNA. There was a good linear relationship between DNA concentrations and luminescence values, and the sensitivity was high. We observed that in solution, the hybridization reaction was not complete and nonspecific binding of the anti-DNA sulphone antibody gave high blank values. Thus, in the case of hybridization experiments using a sulphonated target and a biotinylated probe, only 10 pg of the target was detected.

Similarly, sandwich assays using these two probes (DNA sulphone and biotin), as described by Syvanen *et al.*, (1986), were not very sensitive, showing a detection limit above 50 pg of target.

Detection of polymerase amplification product

Bioluminescent reactions were used to measure the amount of DNA sequenced produced by enzymatic amplification. The specific sequence obtained was labelled at the 5′ end since oligonucleotides were labelled. DNA was captured using streptavidin tubes, which retain biotinylated DNA, and detection was carried out with anti-fluorescein antibody, which bound to the other end of the DNA. The procedure involved three steps, i.e. capture, labelling, and

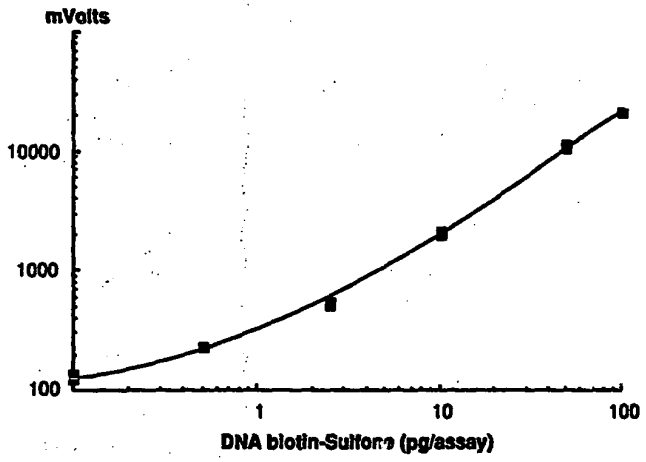

Figure 5. Sandwich assay of a DNA labelled by sulphonation and biotinylation

Table 3. Bioluminescent detection of amplified sequences obtained with four human DNAs and labelled oligonucleotide primers. Oligonucleotide S3 was labelled with fluorescein, and S1, S4 were labelled with biotin

Mismatch bases with primer S4	Amplification with primers S1, S3	Amplification with primers S3, S4
0	31,000	23,000
1	32,000	20,000
2	36,000	15,000
3	30,000	9,540

detection. The amount of sample required for the assay represented less than 1% of the amplified mix after 30 cycles using 1 µl of human DNA. Table 3 shows results obtained with four human DNAs. Amplification was performed with two oligonucleotides present in the four DNAs, and similar luminescence values were obtained. With amplification at 65 °C, using a 17-mer oligonucleotide primer (S4) and 0, 1, 2, or 3 base mismatches, the amounts of amplified sequences decreased rapidly as the mismatches increased. The sequence studied in this experiment is part of the HLA gene and is unique in the human genome. The high sensitivity offered by the amplification reaction and the rapid quantitative determination using bioluminescent assay allow identification of a single mutation occurring in a defined part of the human genome. The use of other labels should improve the method, considering that the antibodies showed relatively low affinity for the fluorescein oligonucleotide derivative.

CONCLUSION

Bioluminescence is a useful method for detecting dehydrogenase immobilized on a filter or in solution. It is fast and can give quantitative results. The sensitivity of the assay was not increased compared to other non-isotopic methods, since nonspecific binding of the label was still very high. We tried to decrease this background noise using small amounts of markers on filters, but to obtain satisfactory results, the incubation time had to be increased considerably. The assay is much more sensitive with the polymerase chain reaction, and when coupled to the bioluminescence detection, the method can be automated.

Another possibility is the use of oligonucleotide or DNA directly labelled by an enzyme. We have coupled thermostable dehydrogenase to oligonucleotide and we are now studying its use in hybridization experiments.

Acknowledgements

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